CELLULAR KINASES INVOLVED IN CYTOMEGALOVIRUS INFECTION

AND THEIR INHIBITION

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Specification

The present invention is in the fields of molecular biology and virology. The
10   present invention is directed to novel methods for treating Cytomegalovirus using
kinase inhibitors.

Background of the invention

15   Human Cytomegalovirus (HCMV) is a highly specific β-herpesvirus. Primary
infection of healthy children and adults is usually asymptomatic, with a minority of
cases developing a mononucleose-like syndrome. In contrast, congenital infection
(U.S. 0.2%-2.2% per live birth; aprox. 40,000 per year) leads to several
neurological defects in 10-15% of infected neonates. Immunocompromised
20   patients represent another host group facing serious disease complications
caused by HCMV infection or reactivation of a persistent infection. Up to 40% of
the AIDS patients, for example, develop retinitis, pneumonitis, gastroenteritis or
disseminated HCMV disease. In addition, allograft recipients (20,000 allograft
transplantations per year in the U.S.) are often infected (or superinfected) by virus
25   from the transplanted organ.

Clinical symptoms in the posttransplant period include prolonged fever,
leukopenia, thrombocytopenia, atypical lymphocytosis, elevated hepatic
transaminases and decreased graft survival. In bone marrow transplantations,
30   HCMV infection is associated with high mortality rates (80-90% for untreated
HCMV pneumonia).

Current approaches to develop therapeutics against Cytomegalovirus (CMV) have
focused on antiviral agents *per se*; for example viral polymerase inhibitors. In fact,
35   high mortality rates have been dramatically reduced by new antiviral agents.
Current CMV therapeutics possess severe drawbacks, however. For example,
Fomivirsen (Vitravene, formerly ISIS 2922) is typically administered by injection
directly into the eye every 2 or 4 weeks. Ganciclovir is available for intravenous

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(Cytovene) or oral administration, and as an implant in the case of retinitis; unfortunately, toxic complications including leukopenia and thrombocytopenia frequently develop. Foscarnet (Foscavir; phosphonoformic acid), another antiviral agent, exhibits considerable renal toxicities and is only available in intravenous

5   form (which is also true for Cidofovir (Vistide), another CMV therapeutic). In addition, CMV replication resumes soon after Ganciclovir and Foscarnet treatment is halted.   Finally, Ganciclovir- and Foscarnet-resistant strains of CMV are emerging.

10   Although treatment of HCMV-induced disease has been improved with these inhibitors of the viral polymerase and preemptive or early antiviral therapy in transplant patients, there is a need in the art for a new class of HCMV therapeutics with better oral bioavailability and reduced toxic effects.   This is especially true in the treatment of retinitis in AIDS patients, where CMV infection

15   must be controlled for long periods of time.

Recent research has revealed how cells communicate with each other to coordinate the growth and maintenance of the multitude of tissues within the human body. A key element of this communication network is the transmission of

20   a signal from the exterior of a cell to its nucleus, which results in the activation or suppression of specific genes. This process is called signal transduction.

An integral part of signal transduction is the interaction of cytokines, their receptors, and intracellular signal transduction molecules. Cytokines serve as

25   messengers that bind to receptors on the surface of a target cell. As a result of the binding, the receptors activate a cascade of downstream signaling molecules, thereby transmitting the message from the exterior of the cell to its nucleus. Signal transduction to the nucleus modulates specific gene expression (i.e., transcription and translation), which results in either the upregulation or

30   downregulation of specific proteins that carry out a particular biological function.

Viral infection disrupts normal signal transduction, which leads to cellular malfunctioning resulting in a disease state. Specifically, interference of HCMV with relevant human primary cells is necessary for the virus to create an

35   environment that allows it to grow and replicate, and in turn cause disease in the infected individual. Current research efforts have failed to elucidate all the specific intracellular signal pathways affected by HCMV infection, however. Discovery of the signal transduction pathways and specific intracellular signal transduction

molecules affected by CMV infection would represent a tremendous advance in the understanding of the induction and progression of CMV infection processes and provide new avenues for the development of a novel class of effective therapeutics for the treatment of CMV.

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Thus, object of the present invention is to provide methods for detecting, preventing and/or treating Cytomegalovirus infection and/or associated diseases, methods for the identification of compounds useful for preventing and/or treating Cytomegalovirus infection and/or associated diseases and for regulating the

10   production of Cytomegaloviruses.

The object of the present invention is solved by the teaching of the independent claims. Further advantageous features, aspects and details of the invention are evident from the dependent claims, the description, and the examples of the

15   present application.

Description of the invention

The present invention is based upon the finding of a group of cellular kinases that

20   are specifically upregulated as a result of CMV infection. The antiviral therapeutic research approach described herein, focuses on discovering the cellular signal transduction pathways involved in viral infection. Identification of the cellular signal transduction molecules, key to viral infection provides for, among other things, novel diagnostic methods, especially assays, and compositions useful

25   therefore, novel targets for antiviral therapeutics, a novel class of antiviral therapeutics, and new screening assays and materials to discover new antiviral agents.

This approach led to the development of a novel microarray platform technology,

30   wherein a microarray of more than 1100 signal transduction cDNAs was developed. This unique microarray technology was used to identify RNA expression patterns (e.g., upregulation or downregulation) unique to CMV infected host cells. Differential display techniques were used to pinpoint those signal transduction molecules useful as targets for drug intervention. Effective

35   manipulation of these virally-controlled intracellular signal transduction pathways can alter (slow or stop altogether) the course of viral growth.

It is now revealed for the first time that the cellular protein kinases RICK (also known as CARDIAK; RIP2), RIP, NIK (also known as HGK; MAP4K4), MKK3 (also known as MEK3), and SRPK-2 are specifically and uniquely upregulated in a cell as a result of CMV infection. These cellular kinases therefore identify novel diagnostic and therapeutic targets for CMV infection.

Surprisingly, it was found that the following human cellular targets are significantly upregulated compared with uninfected human foreskin fibroblasts cells:

target	upregulation
RICK	3.6 fold
RIP	2.6 fold
NIK	4.0 fold
MKK3	2.5 fold
SRPK-2	2.2 fold

Based upon the research work reported herein, one aspect of the present invention is directed to a method, preferably a screening assay, for identifying compounds useful for treating and/or preventing Cytomegalovirus infection and/or diseases associated therewith. Specifically, this assay involves contacting a test compound with one or more cellular kinases selected from the group consisting of RICK, RIP, NIK, MKK3, and SRPK-2, and detecting a change, normally a decrease, in activity of said cellular kinase. This method was used for the identification of the RICK and RIP inhibitors shown below in Table 1 and Table 2.

Another aspect of the invention is directed to a diagnostic method for detecting Cytomegalovirus infection and/or associated diseases in an individual or in cells and/or in cell lysates. This assay involves providing a sample from the individual or providing a sample from said cells, respectively, and detecting activity of one or more cellular kinases selected from the group consisting of RICK, RIP, NIK, MKK3, and SRPK-2. The term "individual" preferably refers to mammals, especially humans or ruminants.

Also described in the present invention are monoclonal or polyclonal antibodies which bind to a cellular kinase selected from the group consisting of RICK, RIP, NIK, MKK3, and SRPK-2.

A further aspect of the present invention relates to a method for preventing and/or treating Cytomegalovirus infection and/or associated diseases in an individual by administering a pharmaceutically effective amount of an inhibitor to said individual, wherein said inhibitor inhibits at least partially the activity of one or more cellular

5   kinases selected from the group consisting of RICK, RIP, NIK, MKK3, and SRPK-2, or wherein said inhibitor inhibits at least partially the production of one or more cellular kinases selected from the group consisting of RICK, RIP, NIK, MKK3, and SRPK-2.

10   As used herein, the term "inhibitor" refers to any compound capable of downregulating, decreasing, reducing, suppressing or inactivating the amount and/or activity of at least one human cellular protein kinase selected from the group consisting of RICK, RIP, NIK, MKK3, and SRPK-2. Generally, said inhibitors, including suicide inhibitors, may be proteins, oligo- and polypeptides,

15   nucleic acids, genes, small chemical molecules, or other chemical moieties. Suitable inhibitors are monoclonal or polyclonal antibodies which bind to at least one cellular kinase selected from the group consisting of RICK, RIP, NIK, MKK3, and SRPK-2.

20   Based on the surprising results reported herein, one aspect of the present invention is directed to a method for regulating the production of Cytomegalovirus in an individual by administering an individual a pharmaceutically effective amount of an inhibitor wherein said inhibitor inhibits at least partially the activity of one or more cellular kinases selected from the group consisting of RICK, RIP, NIK,

25   MKK3, and SRPK-2, or wherein said inhibitor at least partially inhibits the production of one or more cellular kinases selected from the group consisting of RICK, RIP, NIK, MKK3, and SRPK-2.

A similar aspect relates to a method for regulating the production of

30   Cytomegalovirus in cells by administering the cells a pharmaceutically effective amount of an inhibitor wherein said inhibitor inhibits at least partially the activity of one or more cellular kinases selected from the group consisting of RICK, RIP, NIK, MKK3, and SRPK-2, or wherein said inhibitor at least partially inhibits the production of one or more cellular kinases selected from the group consisting of

35   RICK, RIP, NIK, MKK3, and SRPK-2 in the cells.

Yet another aspect of the invention is directed to a method for regulating the expression of at least one cellular kinase selected from the group consisting of

RICK, RIP, NIK, MKK3, and SRPK-2 in an individual comprising the step of administering the individual a pharmaceutically effective amount of an inhibitor wherein said inhibitor inhibits at least partially the transcription of DNA or the translation of RNA encoding one of said cellular kinases.

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A further aspect relates to a method for regulating the expression of at least one cellular kinase selected from the group consisting of RICK, RIP, NIK, MKK3, and SRPK-2 in the cells comprising the step of administering the cells a pharmaceutically effective amount of an inhibitor wherein said inhibitor inhibits at 10 least partially the transcription of DNA or the translation of RNA encoding one of said cellular kinases.

As used herein, the term "regulating expression and/or activity" generally refers to any process that functions to control or modulate the quantity or activity 15 (functionality) of a cellular component. Static regulation maintains expression and/or activity at some given level. Upregulation refers to a relative increase in expression and/or activity. Accordingly downregulation refers to a relative decrease in expression and/or activity. In the present invention, regulation is preferably the downregulation of a cellular component. Downregulation is 20 synonymous with inhibition of a given cellular component's activity.

Beside inhibitors also activators may be useful for treating Cytomegalovirus infection by increasing the activity of at least one of the cellular protein kinases RICK, RIP, NIK, MKK3, and SRPK-2. Thus, a method for preventing and/or 25 treating Cytomegalovirus infection and/or associated diseases in an individual is disclosed. Said method comprises administering a pharmaceutically effective amount of an activator to an individual, wherein said activator activates at least partially the activity of one or more cellular kinases selected from the group consisting of RICK, RIP, NIK, MKK3, and SRPK-2, or wherein said activator 30 activates or stimulates at least partially the production of one or more cellular kinases selected from the group consisting of RICK, RIP, NIK, MKK3, and SRPK-2.

Furthermore, a method for regulating the production of Cytomegalovirus either in 35 cells or in an individual is described. Said methods comprise administering an individual or to cells a pharmaceutically effective amount of an activator wherein said activator activates at least partially the activity of one or more cellular kinases selected from the group consisting of RICK, RIP, NIK, MKK3, and SRPK-2, or

wherein said activator at least partially activates or stimulates the production of one or more cellular kinases selected from the group consisting of RICK, RIP, NIK, MKK3, and SRPK-2.

5　As used herein, the term "activator" refers to any chemical compound which is able to upregulate, increase, activate, or stimulate the activity of at least one human cellular protein kinase selected from the group consisting of RICK, RIP, NIK, MKK3, and SRPK-2 or which is able to upregulate, increase, activate, or stimulate the expression of at least one of said cellular kinases.　Activators

10　comprise proteins, oligo- and polypeptides, nucleic acids, genes, and preferably small chemical molecules, or other chemical moieties.

Still another aspect of the present invention is directed to either a method for regulating the expression of at least one cellular kinase selected from the group

15　consisting of RICK, RIP, NIK, MKK3, and SRPK-2 in an individual or for regulating the expression of at least one of said kinases in cells.　These methods comprise the step of administering the individual or the cells a pharmaceutically effective amount of an activator wherein said activator activates at least partially the transcription of DNA or the translation of RNA encoding one of said cellular kinases.

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Furthermore, oligonucleotides are disclosed which bind to the DNA or RNA encoding a cellular kinase selected from the group consisting of RICK, RIP, NIK, MKK3, and SRPK-2.　Said oligonucleotides can be used as suitable inhibitors within the aforementioned methods.

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Some methods of the present invention identify compounds useful for prophylaxis and/or treatment of Cytomegalovirus infection and/or associated disease by screening a test compound, or a library of test compounds, for its ability to inhibit at least one of the above-mentioned human cellular protein kinases identified

30　herein as characteristically upregulated during HCMV replication.　Using this method the compounds A to E have been identified as RICK inhibitors and the compounds F to H have been identified as RIP inhibitors.　Thus, the use of these compounds as inhibitors of RICK or RIP is disclosed.　Furthermore, these compounds can be used for manufacturing a pharmaceutical composition for

35　prophylaxis and/or treatment of Cytomegalovirus infection and/or diseases associated with Cytomegalovirus infection.

A variety of assay protocols and detection techniques are well known in the art and easily adapted for this purpose by a skilled practitioner. Such methods include, but are not limited to, high throughput assays (e.g., microarray technology, phage display technology), and *in vitro* and *in vivo* cellular and tissue

5   assays.

Thus, some embodiments of the present invention may comprise a solid support useful for detecting Cytomegalovirus infection in a cell or an individual. Preferably the solid support comprises immobilized oligonucleotides, wherein the

10   oligonucleotides are capable of detecting activity of one or more cellular kinases selected from the group consisting of: RICK, RIP, NIK, MKK3, and SRPK-2.

Another aspect of the invention includes a solid support useful for screening compounds useful for treating Cytomegalovirus. Preferred embodiments include

15   a solid support comprising one or more immobilized oligonucleotides, wherein the oligonucleotide(s) encode one or more cellular kinases selected from the group consisting of RICK, RIP, NIK, MKK3, and SRPK-2. In another preferred embodiment, the solid support comprises one or more immobilized cellular kinases selected from the group consisting of: RICK, RIP, NIK, MKK3, and SRPK-

20   2.

Accordingly, another aspect of the present invention is directed to a novel therapeutic composition useful to treat an individual afflicted with Cytomegalovirus comprising one or more inhibitors capable of inhibiting activity of one or more of

25   the cellular kinases selected from the group consisting of RICK, RIP, NIK, MKK3, and SRPK-2. In addition thereto, a novel pharmaceutical composition could comprise at least one inhibitor capable of regulating the production of HCMV by inhibiting the expression of at least one cellular kinase selected from the group consisting of RICK, RIP, NIK, MKK3, and SRPK-2.

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Another group of suitable therapeutic compositions useful for prophylaxis and/or treatment of CMV comprises at least one activator which is able to increase the activity of one or more cellular kinases selected from the group consisting of RICK, RIP, NIK, MKK3, and SRPK-2 or which is capable of increasing the

35   expression of at least one cellular kinase selected from the group consisting of RICK, RIP, NIK, MKK3, and SRPK-2.

Said pharmaceutical compositions may further comprise pharmaceutically acceptable carriers, excipient, diluents, fillers, binders, disintegrants, lubricants, glidents, coloring agents, flavoring agents, opaquing agents, and/or plasticizers.

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Detailed description of the invention

Utilizing microarray technology, a unique microarray of more than 1100 signal transduction cDNAs was developed. This array was used to compare signal transduction mRNA expression patterns (e.g., upregulation or downregulation) in
10   primary human cells before and after infection with HCMV at various timepoints of infection. Interference of the HCMV with the cellular signaling events is reflected in differential gene expression when compared to the uninfected cellular signaling. Results from this novel signal transduction microarray analysis revealed significant upregulation of cellular protein kinases RICK, RIP, NIK, MKK3, and SRPK-2, as
15   unique to CMV infected host cells. These findings were confirmed utilizing conventional Northern and Western blot analyses.

Disclosed herein is the first report describing the role of cellular kinases; RICK, RIP, NIK, MKK3, and SRPK-2 in the signal transduction of CMV viral infection
20   process. As a result of these discoveries, a novel class of compounds, i.e., RICK, RIP, NIK, MKK3, and SRPK-2 inhibitors, are identified as useful for altering the course of CMV infection.

To perform initial tests for compounds that inhibit RICK activity in a cellular assay,
25   RICK was transiently overexpressed in HEK-293 cells, immunoprecipitated and incubated with different concentrations of test compounds before in-vitro kinase assays were performed (Example 10). According to the method for identifying compounds useful for inhibiting the cellular kinase RICK and therefore useful for treating and/or preventing Cytomegalovirus infection and/or diseases associated
30   with Cytomegalovirus infection, a test compound is contacted with the cellular kinase RICK according to the RICK assay protocoll disclosed in example 10. The test compound dissolved in DMSO is added to the RICK assay solution at concentrations between 100 nM and 50 µM. Thereafter, radioactively labeled ATP is added and kinase activity of RICK is determined by detecting the
35   autophosphorylation of RICK via radioactivity measurement. The five compounds listed in the following Table 1 were identified using said method. These compounds showed inhibition of RICK kinase activity with an IC_{50} between about 100 nM and 1 µM and an inhibition of HCMV with an IC_{50} between about 1 and 8

μM, respectively.   The IC$_{50}$ values of HCMV inhibition were obtained by the use of at least one assay protocol selected from a) virus replication assay, b) plaque assay, c) GFP (Green Fluorescent Protein) infection assay, and d) indirect immunofluorescence analysis as disclosed in example 12.   Thus, the five compounds A to E mentioned below and/or pharmaceutically acceptable salts thereof can be used as inhibitors of the cellular protein kinase RICK and as pharmaceutically active compounds for the treatment and/or prophylaxis of HCMV infection. Furthermore, these compounds are suitable for the manufacture of a pharmaceutical composition for prophylaxis and/or treatment of Cytomegalovirus infection and/or diseases associated therewith.

Table 1:   Inhibitors of RICK and HCMV

compound	structure	IC$_{50}$ RICK	IC$_{50}$ HCMV
A AX6548		1 μM	6.8 μM
B AX6558		500 nM	1.4 μM
C AX6549		1 μM	6.2 μM
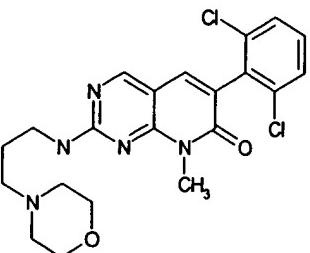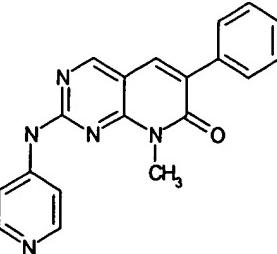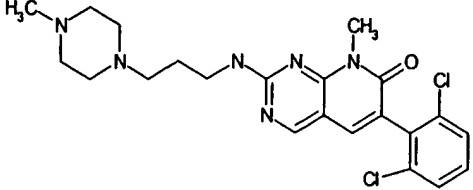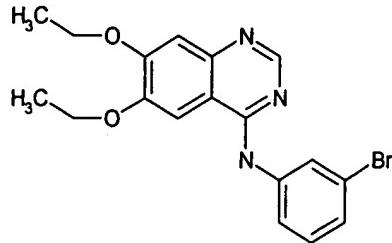
D AX5828		500 nM	7.6 µM
E AX3359		500 nM	5.7 µM
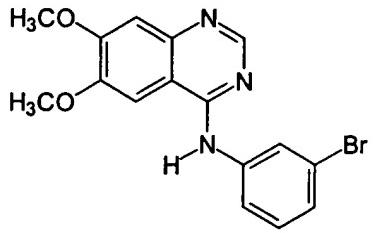
The compounds A to E have the following names:

Compound A: 6-(2,6-Dichlorophenyl)-8-methyl-2-(3-morpholin-4-yl-propylamino)-
8H-pyrido[2,3-d]pyrimidin-7-one;

Compound B: 8-methyl-6-phenyl-2-(pyridin-4-yl-amino)-8H-pyrido[2,3-
d]pyrimidin-7-one;

Compound C: 6-(2,6-Dichlorophenyl)-8-methyl-2-[3-(4-methylpiperazin-1-yl)-
propylamino]-8H-pyrido[2,3-d]pyrimidin-7-one;

Compound D: (3-Bromophenyl)-(6,7-diethoxyquinazolin-4-yl)-amine;

Compound E: (3-Bromophenyl)-(6,7-dimethoxyquinazolin-4-yl)-amine.

From the results observed with the compounds shown in Table 1 it is proved that RICK is an important target for the treatment of HCMV and diseases associated with HCMV infection.   Inhibitors of the human cellular protein kinase RICK may serve as new pharmaceutical substances for prophylaxis and/or treatment of Cytomegalovirus infection and/or diseases associated with CMV infection.

In addition to the chemical validation of RICK described above, a genetic validation of RICK in HCMV infection was performed.   Wildtype and mutated RICK was expressed in HFF cells with a modified Adenovirus as vehicle (Example 9).   The expression of both, wildtype and mutated RICK, caused a dramatic reduction in HCMV replication (cf. Fig. 2).   Also these data confirm RICK as a valuable therapeutic target in HCMV treatment.   As known in the art and as used herein, "RICK" refers to a protein kinase also known as "CARDIAK" and as

"RIP2", is a RIP-like kinase. RICK is essentially characterized as comprising an N-terminal serine-threonine kinase catalytic domain and a C-terminal region containing a caspase-recruitment domain (referred to as "CARD").

5   To perform initial tests for compounds that inhibit RIP activity in a cellular assay, RIP was transiently overexpressed in HEK-293 cells, immunoprecipitated and incubated with different concentrations of test compounds before in-vitro kinase assays were performed (Example 11). In order to identify compounds suitable for inhibiting the cellular kinase RIP and, thus, suitable for treating and/or preventing

10   Cytomegalovirus infection and/or diseases associated with Cytomegalovirus infection the inventive method according to claim 1 was used. A test compound was contacted with the cellular kinase RIP according to the RIP assay protocoll disclosed in example 11. The test compound dissolved in DMSO is added to the RIP assay solution at concentrations between 100 nM and 50 µM. Radioactively

15   labeled ATP was used as co-substrate of RIP and auto-phosphorylation was detected via measurement of incorporation of radioactivity into the RIP protein. Thereafter, phosphorylation rates with and without test compounds were compared. The three compounds listed in the following Table 2 showed inhibition of RIP kinase activity with an IC_{50} between about 5 µM and 10 µM and an

20   inhibition of HCMV with an IC_{50} between about 12 µM and 15 µM, respectively. The IC_{50} values of HCMV inhibition were obtained by the use of at least one assay protocol selected from a) virus replication assay, b) plaque assay, c) GFP infection assay, and d) indirect immunofluorescence analysis as disclosed in example 12.

25   Thus, the three compounds F to H mentioned below and/or pharmaceutically acceptable salts thereof can be used as inhibitors of the cellular protein kinase RIP and as pharmaceutically active compounds for the treatment and/or prophylaxis of HCMV infection. Furthermore, these compounds are suitable for the manufacture of a pharmaceutical composition for prophylaxis and/or treatment

30   of Cytomegalovirus infection and/or diseases associated therewith.

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Table 2:   Inhibitors of RIP and HCMV

compound	structure	IC$_{50}$ RIP	IC$_{50}$ HCMV
F AX 3646 ID 14		5 µM	15 µM
G AX 3597 ID60		10 µM	15 µM
H AX 6333 ID71		5 µM	12 µM
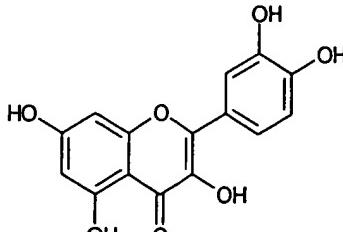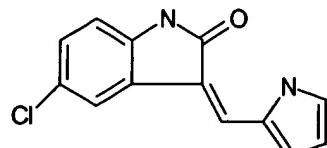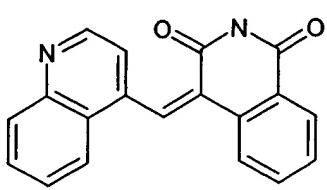
The compounds F to H have the following names:

Compound F: 2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one;
Compound G: 5-Cloro-3-(1H-pyrrol-2-ylmethylene)-1,3-dihydroindol-2-one;
Compound H: 4-Quinolin-4-ylmethylene-4H-isoquinoline-1,3-dione.

From the results observed with the compounds shown in Table 2 it is proved that RIP is an important target for the treatment of HCMV and diseases associated with HCMV infection.   Inhibitors of the human cellular protein kinase RIP may serve as new pharmaceutical substances for prophylaxis and/or treatment of Cytomegalovirus infection and/or diseases associated with CMV infection.

In addition to the chemical validation of RIP described above, a genetic validation of RIP in HCMV infection was performed.   Wildtype and mutated RIP was expressed in HFF cells with a modified Adenovirus as vehicle (Example 9).   The expression of mutated RICK, but not wildtype RIP, caused a dramatic reduction in

HCMV replication (cf. Fig. 1). These data also confirm RIP as therapeutic target in HCMV treatment.

As known in the art and as used herein, "NIK" (Nck-Interacting Kinase; also known
5   as "HGK" or "MAP4K4") refers to an NF-kappaB inducing serine/threonine kinase that interacts with the SH3 domains of Nck (an adaptor protein composed of one SH2 and three SH3 domains, known as a common target for a variety of cell surface receptors). NIK is most homologous to the Sterile 20 (Ste20) family of protein kinases, particularly GCK and MSST1 in that they bind neither Cdc42 nor
10   Rac and contain an N-terminal kinase domain with a putative C-terminal regulatory domain. NIK is reported to promote neurite process formation and mediated anti-apoptotic signaling. NIK expression leads to IKK activation and induced nuclear translocation of NF-kappaB. NIK activates MEK1 phosphorylation and induces the Erk1/Erk2 MAPK pathway. NIK has been shown to be a MEK1-dependent
15   activator of the MAPK pathway (Foehr et al., 2000. J. Biol. Chem. 275, 34021-34024). Overexpression of NIK has been reported to specifically activate the stress-activated protein kinase (SAPK) pathway; possibly upstream of MEKK1, a dominant-negative MEK kinase 1 capable of blocking NIK activation of SAPK (Su et al., 1997. EMBO 16(6):1279-90).
20

As known in the art and as used herein, "MKK3" (MAP kinase kinase 3; also known as "MEK3") refers to a protein kinase known to function in TNF-induced cytokine expression, and specifically phosphorylate and activate p38 MAP kinase (Blank et al., 1996. J. Biol. Chem. 271:5361-5368; Raingeaud et al., 1996. Mol.
25   Cell. Biol. 16(3):1247-55). MKK3 gene disruption has been shown to cause a selective defect in the response of fibroblasts to the proinflammatory cytokine tumor necrosis factor, including reduced p38 MAP kinase activation and cytokine expression; suggesting that the MKK3 protein kinase is a critical component of a tumor necrosis factor-stimulated signaling pathway that causes increased
30   expression of inflammatory cytokines (Wysk et al., 1999. PNAS USA 96(7):3763-8).

As known in the art and as used herein, "SRPK-2"(SR-protein-specific kinase 2) refers to a kinase known to phosphorylate SF2/ASF and believed to regulate the
35   disassembly of the SR family of splicing factors in a tissue-specific manner (e.g., in testis, lung, and brain; Kuroyanagi et al., 1998. Biochem. Biophys. Res. Commun. 242(2):357-64). SRPK-2 is believed to function in spliceosome

assembly and in mediating the trafficking of splicing factors (Wang et al., 1998. J. Cell. Biol. 140(4):737-50; Wang et al., 1999. Genomics 57(2):310-5).

5   In one embodiment, the present invention is directed to a method for treating CMV infection by administering a pharmaceutically effective amount of an inhibitor of one or more of the cellular kinases; RICK, RIP, NIK, MKK3, and/or SRPK-2.

As used herein, a cellular kinase "inhibitor" refers to any compound capable of downregulating, decreasing, suppressing or otherwise regulating the amount
10 and/or activity of a cellular kinase. Inhibition of these cellular kinases can be achieved by any of a variety of mechanisms known in the art, including, but not limited to binding directly to the cellular kinase polypeptide (e.g., a RICK-inhibitor compound binding complex, or substrate mimetic), denaturing or otherwise inactivating the cellular kinase, or inhibiting the expression of the gene (e.g.,
15 transcription to mRNA, translation to a nascent polypeptide, and/or final polypeptide modifications to a mature protein), which encodes the cellular kinase. Generally, cellular kinase inhibitors may be proteins, polypeptides, nucleic acids, small molecules, or other chemical moieties.

20 Yet another aspect of the present invention is directed to pharmaceutical compositions useful for the prophylaxis and/or treatment of an individual afflicted with Cytomegalovirus infection and/or associated diseases.   Said pharmaceutical composition comprises at least one pharmaceutically active compound capable of regulating at least partially the activity or the expression of one human cellular
25 protein kinase selected from the group comprising RICK, RIP, NIK, MKK3, and SRPK-2 and/or capable of regulating the replication of CMV.

As used herein the term "regulating" refers either to the ability of an inhibitor to downregulate, decrease, reduce, suppress, inactivate, or inhibit at least partially the
30 activity of an enzyme, or the expression of an enzyme and the virus replication or to the ability of an activator to upregulate, increase, stimulate, or activate at least partially the activity of an enzyme or the expression of an enzyme.

Suitable examples for inhibitors which are the pharmaceutically active
35 components within the therapeutic compositions are the compounds A to H mentioned in Table 1 and 2.   The compounds 6-(2,6-Dichlorophenyl)-8-methyl-2-(3-morpholin-4-yl-propylamino)-8H-pyrido[2,3-d]pyrimidin-7-one;   8-methyl-6-phenyl-2-(pyridin-4-yl-amino)-8H-pyrido[2,3-d]pyrimidin-7-one;   6-(2,6-

Dichlorophenyl)-8-methyl-2-[3-(4-methylpiperazin-1-yl)-propylamino]-8H-
pyrido[2,3-d]pyrimidin-7-one;   4-[5-(3-Iodophenyl)-2-(4-methanesulfinylphenyl)-
3H-imidazol-4-yl]-pyridine;   (3-Bromophenyl)-(6,7-dimethoxyquinazolin-4-yl)-
amine;   (3-Bromophenyl)-(6,7-diethoxyquinazolin-4-yl)-amine;   2-(3,4-

5   Dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one;   5-Cloro-3-(1H-pyrrol-2-
ylmethylene)-1,3-dihydroindol-2-one;   4-Quinolin-4-ylmethylene-4H-isoquinoline-
1,3-dione;   2,3,7,8-Tetrahydroxychromeno[5,4,3-cde]chromene-5,10-dione;   3-
(1H-Pyrrolo[2,3-b]pyridin-3-ylmethylene)-1,3-dihydropyrrolo[2,3-b]pyridin-2-one
and/or pharmaceutically acceptable salts of these compounds are useful for

10   prophylaxis and/or treatment of Cytomegalovirus infection and/or diseases
associated with Cytomegalovirus infection.

CMV therapeutics may be administered to cells from an individual *in vitro*, or may
involve *in vivo* administration to the individual.   Routes of administration of

15   pharmaceutical preparations to an individual may include inhalation, oral and
parenteral, including dermal, intradermal, intragastral, intracutan, intravasal,
intravenous, intramuscular, intraperitoneal, intranasal, intravaginal, intrabuccal,
percutan, rectal, subcutaneous, sublingual, topical or transdermal application, but
are not limited the these ways of administration.   For instance, the preferred

20   preparations are in administratable form which is suitable for oral application.
These administratable forms, for example, include pills, tablets, film tablets,
coated tablets, capsules, powders and deposits.   Administration to an individual
may be in a single dose or in repeated administrations, and may be in any of a
variety of physiologically acceptable salt forms, and/or with an acceptable

25   pharmaceutical carrier, binder, lubricant, excipient, diluents and/or adjuvant.
Pharmaceutically acceptable salt forms and standard pharmaceutical formulation
techniques are well known to persons skilled in the art (see, for example,
Remington's Pharmaceutical Sciences, Mack Publishing Co.).

30   As used herein, a "pharmaceutically effective amount" of a cellular kinase inhibitor
is an amount effective to achieve the desired physiological result, either in cells
treated *in vitro* or in a subject treated *in vivo*.   Specifically, a pharmaceutically
effective amount is an amount sufficient to inhibit, for some period of time, one or
more of the clinically defined pathological processes associated with the viral

35   infection.   The effective amount may vary depending on the specific kinase
inhibitor selected, and is also dependent on a variety of factors and conditions
related to the subject to be treated and the severity of the infection. For example,
if the inhibitor is to be administered *in vivo*, factors such as the age, weight and

health of the patient as well as dose response curves and toxicity data obtained in preclinical animal work would be among those considered. If the inhibitor is to be contacted with the cells *in vitro*, one would also design a variety of pre-clinical *in vitro* studies to assess such parameters as uptake, half-life, dose, toxicity, etc.

5   The determination of a pharmaceutically effective amount for a given agent is well within the ability of those skilled in the art.

As a result of the discovery of the upregulation of certain cellular kinases as part of the infection process of CMV, a novel diagnostic assay useful for the detecting

10   CMV infection of an individual (or cell) is identified. The diagnostic assay of the present invention involves providing a sample from an individual or providing cells and/or cell lysates, and detecting activity in the sample of one or more cellular kinases selected from the group consisting of RICK, RIP, NIK, MKK3, and SRPK-2. In one embodiment, deviations in the expression levels of one or more of the

15   identified cellular kinases in a test sample compared to a known normal expression levels (e.g., determined from a sample from a healthy individual) will be diagnostic of CMV.

It is apparent to a practitioner in the art that a sample useful for detecting CMV

20   infection, whether of a subject individual or an isolated cell, refers to any cellular extract (including whole cells) from a tissue or body fluid (in the case of an individual) or cellular lysate (in the case of an isolated cell), which contains cellular components representative of cellular activity of one or more of the above-mentioned cellular kinases.

25

It is also apparent to a person of ordinary skill in the art that detection includes any method known in the art useful to indicate the presence, absence, or amount of a detection target. Such methods may include, but are not limited to, any molecular or cellular techniques, used singularly or in combination, including, but not limited

30   to: hybridization and/or binding techniques, including blotting techniques and immunoassays; labeling techniques (chemiluminescent, colorimetric, fluorescent, radioisotopic); spectroscopic techniques; separations technology, including precipitations, electrophoresis, chromatography, centrifugation, ultrafiltration, cell sorting; and enzymatic manipulations (e.g., digestion).

35

Because the present disclosure teaches for the first time the upregulation of a group of cellular kinases specifically involved in the viral infection of CMV, the

present invention is also directed to an assay useful for detecting novel compounds useful for treating CMV infection.

Assays of the present invention identify compounds useful for treating CMV 5 operate by screening a test compound, or library of test compounds, for its ability to inhibit any one or more of the group of cellular kinases identified herein as characteristically upregulated during CMV growth and replication inside a cell. A variety of assay protocols and detection techniques are well known in the art and easily adapted for this purpose by a skilled practitioner. Such assays include, but 10 are not limited to, high throughput assays (e.g., microarray technology, phage display technology), and *in vitro* and *in vivo* cellular and tissue assays.

In a related aspect, it is also an object of the present invention, in view of the discovery of cellular kinases specifically involved in CMV growth in a cell, to 15 provide an assay component specially useful for detecting CMV in an individual (or a cell). Preferably the assay component comprises oligonucleotides capable of detecting activity of one or more of the cellular kinases RICK, RIP, NIK, MKK3, and SRPK-2 in a sample (e.g., by hybridization to mRNA from the sample), immobilized on a solid support. Most preferably the solid support would contain 20 oligonucleotides of sufficient quality and quantity to detect all of the above-mentioned cellular kinases (e.g., a nucleic acid microarray).

Similarly, it is part of the object of the invention to provide an assay component specially useful for screening compounds useful for treating CMV. One preferred 25 assay component comprises oligonucleotides that encode one or more of the cellular kinases RICK, RIP, NIK, MKK3, and SRPK-2, immobilized on a solid support. In another embodiment, the assay component comprises peptide fragments of one or more of the above-identified cellular kinases immobilized on a solid support. Once again the most preferred solid support embodiment would 30 contain polymers of sufficient quality and quantity to detect all of the above-mentioned cellular kinases (e.g., a nucleic acid or a peptide microarray). A variety of assay supports and construction of the same are well known in the art and easily adapted for this purpose by a skilled practitioner (see, for example: Marshall, 1999. "Do-it-yourself gene watching" Science 286:444-447 (including 35 insets); and Service, 2000. "Protein arrays step out of DNA's shadow" Science 289:1673).

It is preferred that mRNA is assayed as an indication of expression. Methods for assaying for mRNA include, but are not limited to, Northern blots, slot blots, dot blots, and hybridization to an ordered array of oligonucleotides. Nucleic acid probes useful for assay of a sample are preferably of sufficient length to
5 specifically hybridize only to appropriate, complementary transcripts. Typically the oligonucleotide probes will be at least 10, 12, 14, 16, 18, 20 or 25 nucleotides in length. In some cases longer probes of at least 30, 40, or 50 nucleotides will be desirable.

10 The cDNA oligonucleotides immobilized on said membrane filter which are used for detecting the up- or downregulation of the above-mentioned human cellular protein kinases by hybridization to the radioactively labeled cDNA probes have the nucleotide sequences listed in table 3.

15 Table 3:   Nucleotide sequences of cDNA-arrays

Human cellular kinase	Sequence of immobilized DNA on arrays (in relation to the respective Acc No)
RICK	914 bp - 2501 bp (AF027706)
RIP	1421 bp - 2617 bp (U50062)
NIK	231 bp - 3077 bp (Y10256)
MKK3	341 bp - 2030 bp (NM_002756)
SRPK-2	1238 bp - 2790 bp (U88666)

The nucleoside sequences of the genes coding for the human cellular protein kinases RICK, RIP, NIK, MKK3, and SRPK-2 listed in Table 3 together with the
20 amino acid sequences of said enzymes can be obtained from NCBI (National Library of Medicine: PubMed; Web address: www.ncbi.nlm.nih.gov/entrez). Sequence protocols of the five cellular protein kinases are attached to this application as a part of the description.

25 The polypeptide product of gene expression may be assayed to determine the amount of expression as well. Methods for assaying for a protein include, but are not limited to, Western blot, immunprecipitation, radioimmunoassay and peptide immobilization in an ordered array. It is understood, however, that any method for specifically and quantitatively measuring a specific protein or mRNA product
30 can be used.

A variety of supports upon which nucleic acids or peptides can be immobilized are known in the art, for example filters, or polyvinyl chloride dishes. Any solid surface to which oligonucleotides or peptides can be bound, either directly or indirectly, either covalently or non-covalently, can be used. A preferred solid support is a

5   microarray membrane filter or a "biochip". These contain particular polymer probes in predetermined locations on the array. Each predetermined location may contain more than one molecule of the probe, but each molecule within the predetermined location has an identical sequence.

10   The present invention incorporates by reference in their entirety techniques well known in the field of molecular biology. These techniques include, but are not limited to, techniques described in the following publications:
Ausubel, F.M. et al. eds., Short Protocols In Molecular Biology (4th Ed. 1999) John Wiley & Sons, NY. (ISBN 0-471-32938-X).

15   Old, R.W. & S.B. Primrose, Principles of Gene Manipulation: An Introduction To Genetic Engineering (3d Ed. 1985) Blackwell Scientific Publications, Boston. Studies in Microbiology; V.2:409 pp. (ISBN 0-632-01318-4).
Miller, J.H. & M.P. Calos eds., Gene Transfer Vectors For Mammalian Cells (1987) Cold Spring Harbor Laboratory Press, NY. 169 pp. (ISBN 0-87969-198-0).

20   Mayer, R.J. & J.H. Walker eds., Immunochemical Methods In Cell and Molecular Biology (1987) Academic Press, London. 325 pp. (ISBN 0-12480-855-7).
Sambrook, J. et al. eds., Molecular Cloning: A Laboratory Manual (2d Ed. 1989) Cold Spring Harbor Laboratory Press, NY. Vols. 1-3. (ISBN 0-87969-309-6).
Winnacker, E.L. From Genes To Clones: Introduction To Gene Technology (1987)

25   VCH Publishers, NY (translated by Horst Ibelgaufts). 634 pp. (ISBN 0-89573-614-4).

The present invention further incorporates by reference in their entirety techniques well known in the field of microarray construction and analysis. These techniques

30   include, but are not limited to, techniques described in the following patents and patent applications describing arrays of biopolymeric compounds and methods for their fabrication: U.S. Pat. Nos. 5,242,974; 5,384,261; 5,405,783; 5,412,087; 5,424,186; 5,429,807; 5,436,327; 5,445,934; 5,472,672; 5,527,681; 5,529,756; 5,545,531; 5,554,501; 5,556,752; 5,561,071; 5,599,895; 5,624,711; 5,639,603;

35   5,658,734; 5,807,522; 6,087,102; WO 93/17126; WO 95/11995; WO 95/35505; EP 742 287; and EP 799 897. Techniques also include, but are not limited to, techniques described in the following patents and patent application describing methods of using arrays in various applications: U.S. Pat. Nos. 5,143,854;

5,288,644; 5,324,633; 5,432,049; 5,470,710; 5,492,806; 5,503,980; 5,510,270; 5,525,464; 5,547,839; 5,580,732; 5,661,028; 5,994,076; 6,033,860; 6,040,138; 6,040,140; WO 95/21265; WO 96/31622; WO 97/10365; WO 97/27317; EP 373 203; and EP 785 280.

5

It is readily apparent to those skilled in the art that other suitable modifications and adaptations of the compositions and methods of the invention described herein are obvious and may be made without departing from the scope of the invention or the embodiments disclosed herein. Having now described the present

10   invention in detail, the same will be more clearly understood by reference to the following examples, which are included for purposes of illustration only and are not intended to be limiting of the invention.

15

Examples

Materials and Methods

5   EXAMPLE 1:

Signal Transduction cDNA Microarray Construction

To study the cellular pathology associated HCMV, a unique microarray of more
10   than 1100 signal transduction cDNAs was created.

In order to manufacture cDNA-arrays on membranes, the following strategy was
employed: cDNAs encoding parts of or full length proteins of interest (referred to
as "target cDNAs") were cloned into the plasmid BLUESCRIPT II KS$^+$ (Stratagene,
15   USA). Large scale purifications of these plasmids were performed according to
standard techniques, and 200μl aliquots (1μg/μl plasmid concentration) were
transferred into appropriate 96 well plates. The plates were then sealed with
sealing tape, incubated for 10 minutes at 95°C, and chilled on ice for 5 minutes.
10μl of 0.6N NaOH were added, and the mix was then stored for 20 minutes at
20   room temperature. Following the incubation at room temperature, 10μl 2.5M Tris-
HCl (Tris-(hydroxymethyl)-aminomethane-hydrochloride) pH7.1 and 20μl 40x SSC
(6M Sodium chloride – 0.6M tri-Sodium citrate buffer) was added.

Target cDNAs were spotted onto Nylon or Nitrocellulose membranes using a
25   BIOGRID (BioRobotics, UK) equipped with a 0.7mm pintool. 200-350ng of
plasmid-encoding target cDNAs were transferred onto the membranes and
crosslinked to the membranes using ultraviolet light (1.2x10^5 μJ/cm^2) treatment.
The arrays were stored for use in subsequent experiments (described below) at
room temperature.
30

EXAMPLE 2:

HCMV Infection
35

To examine the effects of HCMV infection on cellular signal transduction activity,
HCMV-infected cells were generated for comparison to control (i.e., uninfected)
cells.

Primary human foreskin fibroblasts (HFF) were grown close to confluency in MEM medium (Minimum Essential Medium, Life Technologies) supplemented with 20% fetal calf serum at 37°C and 5% CO_2 to obtain ~$6x10^6$ cells per tissue culture flask. Virus adsorption to the cells was performed with the HCMV strain AD169 at

5   different (0.2, 1, and 3) multiplicities of infection (MOI) for 90 minutes in a volume of 5ml at 37°C. The viral inoculum was removed, and cells were cultured in 50ml of MEM medium supplemented with 20% fetal calf serum and 150µg/ml cycloheximid at 37°C and 5%CO_2 for 7, 24, 48, or 72 hours, respectively.

10

EXAMPLE 3:

Isolation and Purification of Poly A$^+$ RNA

15   In order to perform differential expression analysis using the cDNA microarray described in Example 1, RNA extraction and purification on the HCMV-infected and uninfected cells was performed using techniques known in the art.

Briefly, after incubation (for the respective time-intervals) of infected and control
20   cells, cells were washed twice with phosphate buffered saline (PBS) and then trypsinized. Cells were removed from the culture dish by resuspension with PBS. Cells were then sedimented, and directly lysed by repetitive pipetting in 1ml of Tri reagent (Molecular Research Centre, Inc., USA) per $1x10^6$ cells.

25   Cell lysates were stored at room temperature for 5 minutes, and then centrifuged (12,000xg) for 15 minutes at 4°C. The supernatant was mixed with 0.1ml of 1-bromo-3-chloropropane per 1ml of Tri reagent and shaken vigorously. The resultant suspension was stored for 5 minutes at room temperature, and then centrifuged (12,000xg) for 15 minutes at 4°C.
30

Following centrifugation, the colorless upper phase was transferred into new tubes, mixed with 5µl of poly-acryl-carrier (Molecular Research Centre, Inc., USA), and vigorously shaken with 0.5ml of isopropanol per 1ml of Tri reagent. The samples were stored at room temperature for 5 minutes and then centrifuged
35   (12,000xg) for 8 minutes at 4°C. The supernatant was removed and the RNA pellet washed twice with 1ml of 75% ethanol. The pellet was dried and resuspended in RNase-free buffer at a concentration of 1µg RNA per 1µl buffer.

Purification of poly A⁺ RNA from total RNA was performed using the OLIGOTEX system (Qiagen, Germany) following manufacturer's instructions. In brief, 100–200µg of total RNA was brought up to 250µl with RNase-free water, and 250µl of buffer OBB (20mM Tris/HCl pH7.5, 1M NaCl, 2mM EDTA, 0.2% SDS) and 15µl of

5   OLIGOTEX suspension was added. The samples were incubated for 3 minutes at 70°C, and placed at room temperature for 10 minutes. The samples were centrifuged for 2 minutes (12,000xg), and the supernatant removed. The remaining pellet was resuspended in 400µl buffer OW2 (10mM Tris/HCl pH7.5, 150mM NaCl, 1mM EDTA). The suspension was transferred to a spin column

10   (supplied with the system) and centrifuged at 12000xg for 1 minute at room temperature. The spin column was transferred to a new tube and 400 µl of buffer OW2 was applied on the column. The spin column was centrifuged (12,000xg) for 1 minute at room temperature. The spin column was transferred to a new tube and the RNA eluted from the column by the addition of 50µl buffer OEB

15   (5mM Tris/HCl pH7.5) (at 70°C) to the column, resuspension of the Oligotex-resin, and centrifugation (12,000xg) for 1 minute at room temperature.

Any genomic DNA contamination of the RNA preparations was eliminated by enzymatic digestion using DNase I. 6µl of 10x DNase buffer (Promega, USA)

20   and 4µl of RQ-DNase (Promega, USA) were added to 50µl of the RNA-buffer solution, and the reaction mixture was incubated for 15 minutes at 37°C. Stop-buffer (6µl; Promega, USA) was then added, the mixture brought to 200 µl final volume with TE buffer (10mM Tris/HCl, 1mM EDTA), and Phenol/Chloroform extractions were performed twice. The RNA-containing phase was transferred to

25   new reaction tubes and RNA was precipitated using 5M NaCl (final concentration of 0.2M), 1µl poly-acryl-carrier (Molecular Research Centre, Inc., USA) and 500 µl of 100% ethanol. The solution was centrifuged for 10 minutes at 4°C, the RNA pellet washed with 1ml of 80% ethanol, dried, and resuspended in 30µl TE buffer. Poly A⁺ RNA suspension samples were stored at -70°C for use in subsequent

30   experiments.

EXAMPLE 4:

35   Preparation of Radioactively Labeled cDNA Probes from RNA

To prepare test and control samples for microarray analysis, RNA samples isolated and purified from HCMV-infected and control cells (prepared as described

in Example 3) were used to generate radioactively labeled cDNA probe. Many techniques to generate labeled cDNA constructs from cellular RNA extracts are known in the art and applicable to the present invention. Two of those protocols were used in this example to generate radiolabeled cDNA from RNA samples: the
5 first technique involved reverse transcribing cDNA from the RNA sample in the presence of radioactively labelled dATP; the second technique involved first strand cDNA synthesis from the RNA sample, followed by random priming with radioactively labelled dATP.

10 For reverse transcription of cDNA from the RNA sample in the presence of radioactively labelled dATP, 1µg of primer TXN (5'-TTT TTT TTT TTT TTT TXN-3'; SEQ ID NO:1; with T = dTTP; N = dATP, dCTP, dGTP or dTTP; X = dATP, dCTP or dGTP) and total RNA (1 to 15 µg) or poly A$^+$ RNA (20 to 500 ng) were combined in 12 µl bidistilled DEPC-treated H_2O (DEPC: diethylpyrocarbonate) and
15 shaken for 5-15 minutes at 60°C. The mixture was then incubated at 4°C for 2-10 minutes, and centrifuged (10,000xg) for 30 seconds.

After centrifugation, 7µl of a labelling mix (100µCi γ[^{33}P]-ATP (Amersham, UK); vacuum dried and resuspended in 4µl first strand buffer (Life Technologies, USA);
20 2µl 0.1M DTT (dithiothreitol); and 1µl labelling solution -- 4mM dCTP, dGTP, dTTP each and 80 µM dATP final concentration) was added to the RNA solution. 1µl SUPERSCRIPT II reverse transcriptase (Life Technologies, USA) was added and the reaction incubated for 10 minutes at room temperature and then for 60 minutes at 38°C. Following the reaction incubation, 5µl 0.5M EDTA (ethylene
25 diamine tetraacetate) and 25 µl 0.6M NaOH was added to the reaction mixture and shaken vigorously for 30 minutes at 68°C.

Unincorporated nucleotides were removed from the labelling reaction using PROBEQUANT G-50 columns (Amersham, UK). The column (with bottom
30 closure and lid removed) was shaken vigorously and centrifuged (735xg) for 1 minute in an appropriate reaction tube. The column was placed into a new reaction tube, the probe was applied onto the center of the column material and the column was centrifuged (735xg) for 2 minutes. The flow-through was transferred into new reaction tubes and bidistilled H_2O added to 100 µl final
35 volume. 5M NaCl, 1µl poly-acryl-carrier (Molecular Research Centre, Inc., USA) and 250 µl ethanol was added, and the probe precipitated by centrifugation (12,000xg) for 15 minutes. The supernatant was discarded and the pellet dried for subsequent use.

For the alternate labelling technique (random priming with radioactively labelled dATP after first strand cDNA synthesis), the following procedure was followed: 1μg primer TXN (see above) was added to 20-500ng of poly A$^+$ RNA in 12 μl final volume, incubated for 5 minutes at 60°C, followed by an addition incubation for 2-10 minutes on ice. The mix was centrifuged (12,000xg) for 30 seconds, and 4μl of first strand buffer (Life Technologies, USA), 2μl 0.1M DTT, 1μl 10mM dNTP and 1μl SUPERSCRIPT II reverse transcriptase (Life Technologies, USA) was added. The reaction was incubated for 10 minutes at room temperature, followed by an additional incubation for 60 minutes at 38°C. Following the reaction incubation, 5μl 0.5M EDTA and 25 μl 0.6M NaOH was added to the reaction mixture and shaken vigorously for 30 minutes at 68°C.

Unincorporated nucleotides were removed as described above; however, the final pellet was resuspended in 30μl bidistilled H_2O.

15 μl of the resuspended cDNA solution was transferred to new reaction tubes, incubated for 5 minutes at 95°C, chilled on ice for 5 minutes, and centrifuged for 30 seconds. Following manufacturer's instructions accompanying the Random Primers DNA Labelling system (Life technologies, USA), 15 μl buffers mixture, 2μl of each dCTP, dGTP and dTTP (provided with the system) were added to the cDNA. 5μl γ[^{33}P]-ATP (Amersham, UK) was added and the mixture adjusted to 49μl final volume with bidistilled H_2O. The reaction was started by addition of 1μl Klenow enzyme (supplied with the system), and incubated for 60 minutes at 25°C 5μl. Stop solution (provided with the system) was added and unincorporated nucleotides were removed by column purification as described above.

EXAMPLE 5:

Hybridization of Labeled cDNA Probe to cDNA Array

To screen HCMV-infected cells compared to uninfected cells for differential activation of cellular signal transduction, labeled cDNA probes (generated according to Example 4) were exposed to a signal transduction cDNA microarray (generated as described in Example 1) following hybridization techniques known in the art.

Sample pellets from Example 4 were resuspended in 10µl C_0T DNA (1µg/µl, Roche Diagnostics, Germany), 10µl yeast tRNA (1µg/µl Sigma, USA) and 10µl poly A (1µg/µl, Roche Diagnostics, Germany). Herring sperm DNA (to a final concentration of 100µg/ml), 5µl 10% SDS (Sodiumdodecylsulfate), and 25 µl 20x

5   SSPE was added, and adjusted 100µl final volume with bidistilled H_2O. The mix was incubated for 5 minutes at 95°C, centrifuged (10,000xg) for 30 seconds, and vigorously shaken for 60 minutes at 68°C. A 1µl aliquot of the probe was used to measure the incorporation of radioactive dATP with a scintillation counter. Probes with at least a total of $20x10^6$ cpm were used for the screen assay.

10

Arrays were prehybridized in hybridization solution for at least 30 minutes in a roller bottle oven at 42°C. Following prehybridization, radiolabelled probe was added to the hybridization solution and hybridization was continued for 20-40 hours.

15

Following hybridization, the probe was discarded and the array subjected to a series of washes. Initially the arrays were washed twice in wash solution A (2xSSC) in the roller oven at room temperature. Wash solution A was then replaced with wash solution B (2x SSC, 0.5% SDS), preheated to 60°C, and

20   arrays were washed twice for 30 minutes at 60°C. Wash solution B was then replaced with wash solution C (0.5x SSC, 0.5% SDS), preheated to 60°C, and arrays were washed twice for 30 minutes at 60°C.

The moist arrays were wrapped in airtight bags and exposed for 8-72 hours on

25   erased phosphoimager screens (Fujifilm, Japan).

EXAMPLE 6:

30   Signal Transduction cDNA Array Analysis

To demonstrate differential activation of cellular signal transduction in HCMV-infected cells compared to uninfected cells, hybridized cDNA arrays from infected and uninfected samples were analyzed.

35

Exposed phosphoimager screens (from Example 5) were scanned with a resolution of 100µ and 16bits per pixel using a BAS-1800 (Fujifilm, Japan). The data were imported into the computer program, ARRAYVISION (Imaging

Research, Canada), and analyzed according the computer program's specification. Hybridization signal strength is indicative of the quantity of RNA molecules present in the probe. Differentially expressed genes were identified according to the ratio of signal strength after normalization to the overall intensity
5   of the arrays.

Signal transduction cDNA microarray analysis of radiolabelled cDNA-probes from HCMV-infected (strain AD169) versus non-infected primary human foreskin fibroblasts to cDNA-arrays revealed significant upregulation of the cellular kinase
10 cDNAs:
    RICK (2-fold at 3 hours post infection; 3.6-fold at 7 hours post infection);
    RIP (2.6-fold at 3 hour post infection; 2.2-fold at 24 hour post infection);
    NIK (4-fold at 7 hour post infection);
    MKK3 (2-fold at 3 hour post infection; 2.5-fold at 7 hour post infection); and
15 SRPK-2 (2.2-fold at 7 hour post infection)
compared to uninfected human foreskin fibroblasts cells.

20 EXAMPLE 7:

Northern Blot Analysis

To confirm the results of the microarray analysis of Example 6, northern blot analysis was performed according to techniques well known in the art.
25

HCMV-infected and uninfected cells (from Example 2) cells were pelleted and the total RNA was prepared as follows: Following centrifugation and removal of the supernatant, cells were lysed in 1ml of Trizol reagent (ready-to-use-reagent from Gibco-BRL) per $1.5 \cdot 10^6$ cells. The Tri reagent/cell lysate was transferred to an
30 eppendorff tube and centrifuged (13,000rpm) for 15 minutes at 4°C. The supernatant was transferred to a new eppendorff tube and 0.1ml of BCP (1-bromo-3-chlorpropane) for each ml of Tri reagent was added. Samples were vortexed for 15 seconds, incubated for 5 min at room temperature, and then centrifuged (13,000rpm) for 15 minutes at 4°C. The upper aqueous phase was
35 transferred to a new eppendorff tube, 0.5 ml isopropanol was added for each ml of Tri reagent (Molecular Research Center, Inc., USA), vortexed, and incubated for additional 8 min at room temperature, and centrifuged (13,000rpm) for 10 min at 4°C. The supernatant was aspirated, and the precipitated RNA was washed twice

with ice-cold 75% ethanol and air-dried. The RNA pellet was resuspended in 50µl Tris-HCl pH 7.5.

5 The quantity of the RNA for each sample was determined by UV-spectroscopy, and the quality was determined via gel electrophoresis on a formaldehyde-containing 1.2% agarose gel.

RNA samples of 10µg each were size-fractionated by 1.2% formaldehyde agarose gel electrophoresis and transferred to synthetic membrane filters (Hybond N, 10 Amersham) with 20XSSC (1 X SSC is 150 mM NaCl, 15 mM $C_6H_5Na_3O_7$ x $2H_2O$, pH 7.0) overnight. RNA was immobilized to the filter using UV-light for crosslinking (120 mJ/cm^2 for 25 seconds).

Membrane filters were firstly prehybridized for 4 hours at 65°C in a 15 prehybridization solution containing 5 X SSC, 10 X Denhardt's solution (1 X Denhardt's solution is 0.02% bovine serum albumine, 0.02% polyvinyl pyrrolidone, 0.02% ficoll), 20mM sodium phosphate, pH 7.0, 7% SDS, 100µg/ml sonicated salmon sperm DNA, and 100µg/ml. Hybridization was performed at 65°C in the prehybridization buffer containing 10% dextran sulphate, plus added radiolabelled 20 probe for 16 hours.

Membrane filters were hybridized to oligonucleotide probes specific for a particular cellular kinase identified in Example 6. Probes sequences included the following:

25 Table 4:

Cellular Kinase	cDNA Probe Sequence	SEQ ID NO:
NIK	5'- GTC CTG GAG GGC TCT TTT TGA TGA AAC C – 3'	2
RIP	5'- GTG CTC AAT GCA GTT GGG CCC CTT GTA CAC-3'	3
RICK	5'- GTC GAG CAG CGG AGT GTG GAT GTG CAG – 3'	4

The oligonucleotides were radiolabelled at their 3′ ends with (alpha-32P) deoxyadenosine 5′- triphosphate (^{32}P-α-dATP) (Amersham) employing the 30 Terminal Transferase kit (Roche) following manufacturer's instructions.

Unincorporated ^{32}P-α-dATP nucleotides were removed similar to the protocol described in Example 4: After vortexing the PROBEQUANT Sephadex G-50

(Amersham, UK) column (with bottom closure and lid removed), the column was placed in a 2 ml tube and centrifuged for 1 minute at 735xg. The column was placed in a new 1.5ml eppendorff tube (without a cap), and the radioactive probe was pipetted carefully on the center of the preformed resin. Centrifugation (735xg)

5   for 2 minutes effectively removed the unincorporated ^{32}P-α-dATP nucleotides.

Hybridized filters were washed once in 5% SDS, 3 X SSC, 10 X Denhardt's solution, 20mM sodium phosphate, pH 7.0 for 30 min at 65°C. A second wash step followed in 1 X SSC, 1% SDS at 65°C for 30 min.

10

Filters were exposed at –80°C to Kodak XAR-5 films using intensifying screens.

Northern blot analysis confirmed upregulation of cellular kinase mRNA: RICK, RIP, and NIK in HCMV-infected cells compared to uninfected cells, consistent with

15   results obtained from microarray analysis.

EXAMPLE 8:

20   Western Blot Analysis

To further confirm the results of the microarray analysis of Example 6 and northern blot analysis of Example 7, western blot analysis was performed according to techniques well known in the art.

25

HCMV-infected and uninfected cells (from Example 2) were pelleted and polypeptide extracts prepared as follows: Infected and uninfected cell samples (from various time intervals) were lysed with 420 μl of lysis buffer (20mM Hepes (N-[2-hydroxyethyl]piperazine-N´-[2-ethansulfonic acid]) pH7.5, 150 mM NaCl, 1%

30   TRITON X-100 (t-octylphenoxypolyethoxyethanol), 10% glycerol, 1mM PMSF (phenylmethylsulfonyl fluoride), 10μg/ml Aprotinin, 1mM ortho-vanadat) on ice. Lysed cells were cleared from debris by centrifugation (15 minutes, 13000 rpm, 4°C), dissolved in 1x Laemmli buffer, denatured for 5 minutes at 100°C and submitted to SDS-PAGE (gradient gel 7% - 12%).

35

Gels were blotted onto nitrocellulose filters (Amersham, UK) for 3 hours (0.8mA/cm2). Detection of expression of the identified host cell kinases was performed using the following target specific antibodies: OPA1-01023 polyclonal

rabbit anti-RICK antibody (Dianova); H-207 polyclonal rabbit anti-RIP antibody (Santa Cruz Biotechnology); I-20 polyclonal rabbit anti-MKK3 antibody (Santa Cruz Biotechnology); S80620 murine anti-SRPK2 antibody (Transduction Laboratories); anti-NIK rabbit serum (generated by SIGMA Genosys

5   Biotechnologies using the NIK-peptide 5′-CNPTNTRPQSDTPEIRKYKKRFN-3′, SEQ ID NO:5, for immunization). All antibodies were used according to the manufacturer's instructions. Detections were performed with the ECL Kit (Amersham, UK).

10   Western blot analysis confirmed the transcriptional upregulation of infected host cell kinase mRNAs resulted in increased expression of the respective proteins:
A single ~60kDa band representing RICK was upregulated between 7-24 hours post HCMV infection;
A single ~74kDa band representing RIP was upregulated between 7-72 hours post

15   HCMV infection;
A single ~135kDa band representing NIK was upregulated between 24-72 hours post HCMV infection;
A single ~35kDa band representing MKK3 was upregulated between 7-72 hours post HCMV infection; and

20   A single ~115kDa band representing SRPK2 was upregulated between 24-72 hours post HCMV infection.

25   EXAMPLE 9:

Genetic Validation

HFF cells were infected with Adenovirus expressing various kinase constructs at different particles per cell ratios (p/c). The adenovirus used here were all E1, E3

30   defective derivatives of adenovirus type 5 (reviewed in Russell WC (2000) Update on adenovirus and its vectors. J Gen Virol. 81:2573-604). Briefly, the cDNA of interest was cloned into a transfer plasmid bearing the CMV IE promoter enhancer (IE: immediate early) and the rabbit beta-globin intron/polyadenylation signal. This expression cassette was inserted into a bacterial plasmid borne-

35   adenovirus genome using recombination in bacteria (Chartier C., E. Degryse, M. Gantzer, A. Dieterle, A. Pavirani, and M. Mehtali. 1996. Efficient generation of recombinant adenovirus vectors by homologous recombination in Escherichia coli. J. Virol. 70:4805-4810.). Virus was amplified in HEK 293 cells and purified

from cell lysates using CsCl density gradient centrifugation as described (Cotten, M., Baker A., Birnstiel M.L., Zatloukal, K., Wagner, E. (1996) Adenovirus polylysine DNA conjugates. in Current Protocols in Human Genetics, Eds. N. C. Dracopoli, J. L. Haines, B.R. Korf, D.T. Moir, C.C. Morton, C.E. Seidman, J.G.

5   Seidman, D.R. Smith; John Wiley and Sons, Inc. New York. pp. 12.3.1-12.3.33.). The control viruses AdJ5 was previously described (Glotzer J.B., Saltik M., Chiocca S., Michou A.I., Moseley P. and Cotten M. (2000) Activation of heat-shock response by an adenovirus is essential for virus replication. Nature 407:207-11).

10

Two days after plating HFF cells, cultures were infected with CMV strain Ad169-GFP. Replication of CMV was estimated after one week (7 dpi) utilizing the GFP-signal expressed as GFP counts.

15   Fig. 1 shows the reduction rates in HCMV replication of HHF cells pre-infected with Adeno virus containing the RIP wildtype sequence (AdRIPwt; 1 – 4) and a RIP inactive mutant (AdRIPkr; 5 – 8).

No HCMV-infection resulted in hardly any signal (mock, 13), while infection with
20   HCMV yielded in about 13.000 GFP counts (AD169-GFP; 14). Pre-infection with increasing amounts of control Adeno virus (AdliteJ5) caused a slight reduction in HCMV replication (9 – 12). There was a clear difference, when HFF cells were pre-infected with Adeno virus containing the RIP wildtype sequence (AdRIPwt; 1 – 4) and a RIP inactive mutant (AdRIPkr; 5 – 8). The lysine (K) at amino acid
25   position 45 is mutated to an arginine (R), which renders the kinase inactive. This mutation was introduced into the human RIP cDNA utilizing the QuikChange™ Site-directed Mutagenesis Kit (Stratagene, CA, USA) according to the instructions of the manufacturer. Expression of the mutated RIP kinase efficiently blocked HCMV replication (5 – 8), while the wildtype sequence was less potent in doing so
30   (1 – 4).

Fig. 2 shows the reduction rates in HCMV replication of HHF cells pre-infected with Adeno virus containing the RICK wildtype sequence (AdRICKwt) and two RICK inactive mutants (AdRICKkr and AdRICKdn). In one construct (AdRICKkr),
35   the lysine (K) at position 47 is mutated to an arginine (R). In the other construct (AdRICKdn), the aspartate at position 146 is mutated to an asparagine. Both changes in sequence render the kinase inactive. The mutations were introduced

into the human RICK cDNA utilizing the QuikChange™ Site-directed Mutagenesis Kit (Stratagene, CA, USA) according to the instructions of the manufacturer.

5 Similar experiments as described for RIP (Example 9, Fig. 1) were also performed with RICK. No HCMV-infection resulted in hardly any signal (Mock + Mock), while infection with HCMV yielded in about 7.000 GFP counts (Mock + AD169-GFP). Pre-infection with increasing amounts of control Adeno virus (AdliteJ5, from 111 to 3000 particles per cell) caused a slight reduction in HCMV replication. There was a clear difference, when HFF cells were pre-infected with Adeno virus containing 10 the RICK wildtype sequence (AdRICKwt and two RICK inactive mutants (AdRICKkr and AdRICKdn). All three RICK constructs efficiently reduced HCMV-replication.

15 EXAMPLE 10:

RICK-Kinase Assay

To obtain active RICK kinase the human RICK-cDNA was fused with a DNA 20 sequence coding for the HA-tag and cloned into the eucaryotic expression vector pcDNA3 (Invitrogene). This construct was transfected into human embryonic kidney cells (HEK 293) via the calcium-phosphate co-precipitation method. One day after transfection medium was replaced by fetal calf serurum-free medium and two days after transfection cells were washed with PBS and harvested and 25 lysed in RIPA-buffer (150 mM NaCl, 1 mM EDTA, 1% Tritron X-100, 1% Na-desoxycholate, 0.1 % SDS, 10 mM Tris-HCl pH 7.5). The RICK-HA fusion protein was immunoprecipitated from 250 µl cleared lysate (i.e. lysate of one well of a six-well plate) utilizing an anti-HA antibody from Roche Pharmaceuticals and Protein A sepharose. After addition of 500 µl of HNTG-buffer (50 mM HEPES pH 7.5, 30 150 mM NaCl, 1 mM EDTA, 10% glycerine, 0.1% Triton X-100) the sample was rotated for 3 hrs at 4°C. After washing the immunoprecipitate twice with 0.5 ml HNTG-buffer and twice with 0.5 ml assay-buffer (25 mM Tris-HCl pH 7.5, 3 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.5 mM DTT and 50 mM NaCl), the kinase reaction was performed directly on the beads in 40 µl assay buffer containing 2.5 µCi $\gamma[^{33}P]$-35 ATP and various concentrations (between 100 nM and 50 µM) of compounds of Table 1. After 30 min at 30°C, the reaction was stopped by addition of 40 µl 3X Laemmli-buffer (16% glycerol, 1.01M ß-mercaptoethanol, 5% SDS, 200mM

Tris/HCl pH 6.8, 8% bromphenolblue). Phosphorylation products were analyzed by SDS-PAGE and auroradiography (x-ray film and phosphor imager).

5 EXAMPLE 11:

RIP-Kinase Assay

To obtain active RIP kinase the human RIP-cDNA was fused with a DNA
10 sequence coding for the HA-tag and cloned into the eucaryotic expression vector pcDNA3 (Invitrogene). This construct was transfected into human embryonic kidney cells (HEK 293) via the calcium-phosphate DNA co-precipitation method. Two days after transfection cells were washed with PBS and harvested and lysed in lysis-buffer (150 mM NaCl, 1 mM EDTA, 1% Tritron X-100, 20 mM Tris-HCl pH
15 7.5 and freshly added: 30 mM NaF, 10 µg/ml Aprotinine, 10 µg/ml Leupeptine, 2 mM Na-pyrophosphate). The RIP-HA fusion protein was immunoprecipitated from 250 µl cleared lysate (i.e. lysate of one well of a six-well plate) utilizing an anti-HA antibody from Roche Pharmaceuticals and Protein A sepharose. The sample was rotated for 3 hrs at 4°C. The immunoprecipitates were washed twice
20 with 0.75 ml lysis-buffer, twice with 0.75 ml high salt-buffer (1 M NaCl, 1 mM EDTA, 1% Tritron X-100, 20 mM Tris-HCl pH 7.5 and freshly added: 30 mM NaF, 10 µg/ml Aprotinine, 10 µg/ml Leupeptine, 2 mM Na-pyrophosphate), twice with 0.75 ml lysis-buffer and twice 0.75 ml with kinase assay buffer (10 mM $MgCl_2$, 10 mM $MnCl_2$, 10 mM benzamidine, 0.5 mM EDTA). The kinase reaction was
25 performed directly on the beads in 40 µl kinase assay buffer containing 2.5 µCi $\gamma[^{32}P]$-ATP and various concentrations (between 100 nM and 50 µM) of compounds of Table 2. After 30 min at 30°C, the reaction was stopped by addition of 40 µl 2X Laemmli-buffer. Phosphorylation products were analyzed by SDS-PAGE and autoradiography (x-ray film and phosphor imager).
30

EXAMPLE 12:

Virus Replication Assay
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Cell culture and virus
Primary human foreskin fibroblasts (HFF) were cultivated in MEM containing 5% (v/v) fetal calf serum. Infection analysis was restricted to cell passage numbers

below twenty. Human cytomegalovirus strain AD169 (ATCC) was grown in HFF cells and quantitated for infectivity by the plaque reduction assay. Aliquots were stored at –80°C.

5 Construction of recombinant cytomegalovirus

For construction of a recombination vector, two linker sequences were inserted into the pBlueScribe vector pBS+ (Stratagene): the first contained restriction sites for NheI, SpeI, PacI and BglII followed by a loxP sequence (ATAACTTCGTATAGCATACATTATACGAAGTTAT) and was introduced into

10 PstI/XbaI sites of the vector; the second contained another loxP sequence followed by restriction sites HpaI, ClaI and PmeI and was introduced into BamHI/Asp718 sites. A gene cassette comprising of a "humanized" version of the ORF coding for GFP (gfp-h) under the control of the HCMV enhancer/promoter and the Ptk/PY441 enhancer-driven neoR selection marker was excised from plasmid pUF5 (Zolotukhin

15 et al., 1996, J. Virol. 70, 4646-4654) and inserted into the recombination vector via BglII sites.

At the 5' and 3'-positions of this loxP-flanked gene cassette, two HCMV sequences with homology to the gene region containing the open reading frames US9 and

20 US10 were inserted. For this, viral sequences were amplified from template pCM49 (Fleckenstein et al., 1982, Gene 18, 39-46) via PCR in a 35-cycle program (denaturation 45 sec at 95°C, annealing 45 sec at 55°C and elongation 2 min at 72°C) by the use of Vent DNA polymerase (New England Biolabs). A US10-specific sequence of 1983 bp in length was generated using primers

25 US10[200900]SpeI (GCT**CACTAGT**GGCCTAGCCTGGCTCATGGCC) and US10[198918]PacI (GTCC**TTAATTAA**GACGTGGTTGTGGTCACCGAA) and inserted at the vector 5' cloning position via SpeI/PacI restriction sites (see boldprint). A US9-specific sequence of 2010 bp was generated using primers US9-3'PmeI (CT**GGGTTTAAAC**GACGTGAGGCGCTCCGTCACC) and US-5' ClaI

30 (TTGCAT**CGAT**ACGGTGTGAGATACCACGATG) inserted at the vector 3' cloning position via PmeI/ClaI restriction sites.

The resulting construct pHM673 was linearized by the use of restriction enzyme NheI and transfected into HEF cells via the electroporation method using a Gene

35 Pulser (Bio-rad; 280 V, 960 µF, 400 Ω). After 24 h of cultivation, cells were used for infection with 1 PFU/ml of HCMV strain AD169. Selection with 200 µg/ml G418 was started 24 h post infection. Following 3 weeks of passage in the presence of G418, GFP fluorescence could be detected in most of the infected cells. Plaque assays

were performed with infectuous culture supernatant on HFF cells and single virus plaques were grown by transfer to fresh HFF cells cultured in 48-well plates. DNA was isolated from cells of 32 fluorescence-positive wells and confirmed for the presence of recombinant virus by PCR. For this, primers US9[198789]

5 (TGACGCGAGTATTACGTGTC)   and   US10[199100] (CTCCTCCTGATATGCGGTT) were used resulting in an amplification product of 312 bp for wild-type AD169 virus and approximately 3.5 kb for recombinant virus.

Plaque assay

10 HFF cells were cultivated in 12-well plates to 90-100% confluency and used for infection with dilutions of virus-positive cell culture supernatants. Virus inoculation was performed for 90 min at 37°C under occasional shaking before virus was removed and the cell layers were rinsed with PBS. Overlays of MEM 5% (v/v) fetal calf serum and 0.3% (w/v) agarose were added to each well and all samples were

15 incubated at 37°C in a 5% CO_2 atmosphere for approximately 12 days. Finally, overlays were removed and the formation of foci was visualized by staining with 1% crystal violet in 20% ethanol for 1 min. After repeated rinsing with PBS, plates were air-dried at room temperature and plaque numbers were counted with a light microscope. For the recombinant AD169-GFP virus, quantification of plaque

20 assays could also be performed without crystal violet staining by a direct counting of the amount of green fluorescent plaques using fluorescence microscopy.

Antiviral compounds

The reference compounds used for antiviral studies, ganciclovir (GCV, Cymeven),

25 foscarnet sodium (FOS, Foscavir) and cidofovir (CDV, Vistide) were purchased from Syntex Arzneimittel (Aachen, Germany), Sigma-Aldrich (Germany) and Pharmacia & Upjohn S.A. (Luxembourg), respectively. Stocks were prepared in aequeous solution and stored at –20°C. The test compounds were dissolved in DMSO and aliquots were stored at –20°C.

30

GFP infection assay

HFF cells were cultivated in 12-well plates to 90-100% confluency and used for infection with 0,5xTCID$_{50}$ of AD169-GFP virus. Virus inoculation was performed for 90 min at 37°C with occasional shaking before virus was removed and the cell

35 layers were rinsed with PBS. Infected cell layers were incubated with 2 ml of MEM containing 5% (v/v) fetal calf serum and optionally of the respective test substances or DMSO as control. Infected cells were incubated at 37°C in a 5% CO_2 atmosphere for 7 days and harvested by trypsination and centrifugation. 200 µl of

lysis buffer (25 mM Tris pH 7.8, 2 mM DTT, 2 mM trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 1% Triton X-100, 10% glycerol) was added to each cell pellet and lysis was achieved by incubation for 10 min at 37°C followed by a 30-min incubation at room temperature on a shaker. Lysates were centrifuged for 5 min at

5   15.000 rpm in an Eppendorf centrifugue to remove cell debris. Supernatants were transferred to an opaque 96-well plate for automated measuring of GFP signals in a Victor 1420 Multilabel Counter (Wallac). GFP units were converted to percent inhibition values relative to DMSO controls (set at 100% GFP expression).

10   Indirect immunofluorescence analysis

Cells were either grown on Lab-Tek Permanox slides (Nunc) or harvested from 6-well plates, spotted onto glass slides with marked rings (Medco) and fixed by a 15-min treatment with 3% formaldehyde in PBS followed by permeabilization for 15 min in 0.1% Triton X-100 in PBS at room temperature. Blocking was achieved by

15   incubation with Cohn Fraction II/III of human gamma-globulin (Sigma; 2 mg/ml) for 30 min at 37°C. The IE1/IE2-specific primary antibody MAb810 (Chemicon International, Inc. CA, USA; dilution 1:10.000) was incubated for 90 min, the secondary antibody (tetramethyl rhodamine [TRITC]-coupled anti-mouse antibody, Dianova, dilution 1:100) for 45 min at 37°C before analysis by fluorescence

20   microscopy. In addition to indirect TRITC staining of IE1/IE2 proteins, GFP signals could be detected diretcly via the fluorescence isothiocyanate (FITC) channel. Nuclear counterstaining was carried out using Vectashield mounting medium including DAPI (Vector Laboratories, Burlingame, CA).

25